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Effect of polyamine depletion on caspase activation: a study with spermine synthase-deficient cells

Claudio STEFANELLI*[1], Carla PIGNATTI*, Benedetta TANTINI*, Monia FATTORI*, Ivana STANIC*, Caroline A. MACKINTOSH†, Flavio FLAMIGNI*, Carlo GUARNIERI*, Claudio M. CALDARERA* and Anthony E. PEGG†

*Department of Biochemistry 'G. Moruzzi', University of Bologna, Via Irnerio 48, I-40126 Bologna, Italy, and †Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, The Milton S. Hershey Medical Centre, Hershey, PA 17033, U.S.A.

Activation of the caspase proteases represents a central point in apoptosis. The requirement for spermine for the processes leading to caspase activation has been studied in transformed embryonic fibroblasts obtained from gyro (Gy) mutant male mice. These cells lack spermine synthase activity and thus provide a valuable model to study the role of spermine in cell processes. Gy fibroblasts do not contain spermine and have a higher spermidine content. However, when compared with fibroblasts obtained from normal male littermates (N cells), Gy fibroblasts were observed to grow normally. The lack of spermine did not affect the expression of Bcl-2, and caspases 3 and 9 were activated by etoposide in both N and Gy cells, indicating that spermine is dispensable for caspase activation. Spermine deficiency did not significantly influence caspase activity in cells treated with etoposide, cycloheximide or staurosporine, but sensitized the cells to UV irradiation, which triggered significantly higher caspase activity in Gy cells compared with N cells. α-Difluoromethylornithine (DFMO), an inhibitor of polyamine synthesis that is able to deplete cells of putrescine and spermidine, but usually does not influence spermine content, was able to produce a more complete polyamine depletion in Gy cells. This depletion, which included spermine deficiency, dramatically increased caspase activation and cell death in Gy fibroblasts exposed to UV irradiation. On the other hand, in either N or Gy cells, DFMO treatment did not influence caspase activity triggered by staurosporine, but inhibited it when the inducers were cycloheximide or etoposide. In Gy cells depleted of polyamines by DFMO, polyamine replenishment with either spermidine or spermine was sufficient to restore caspase activity induced by etoposide, indicating that, in this model, polyamines have an interchangeable role in supporting caspase activation. Therefore, spermine is not required for such activation, and the effect and specificity of polyamine depletion on caspase activity may be very different, depending on the role of polyamines in the specific death pathways engaged by different stimuli. Some inducers of apoptosis, for example etoposide, absolutely require polyamines for caspase activation, yet the lack of polyamines, particularly spermine, strongly increases caspase activation when induced by UV irradiation.

Key words: cycloheximide, D,L-α-difluoromethylornithine, etoposide, gyro, staurosporine, UV-induced damage.

INTRODUCTION

Apoptosis is a highly regulated process, essential for normal tissue development and homoeostasis [1,2]. Importantly, induction of apoptosis is also a main mechanism involved in the killing of tumour cells by anticancer treatments [3]. The central point in apoptosis is represented by the activation of a family of proteases, the caspases, that execute the cell-death programme [4]. Two main pathways leading to caspase activation are now recognized [2,4]. The first involves the activation of death receptors by extracellular signals, whereas the second pathway involves the release of caspase-activating molecules, such cytochrome *c*, from the mitochondria into the cytosol.

Although contrasting, cell death and cell proliferation appear to be linked [5]. Indeed, during oncogenic transformation, increased cell death is observed. One of the possible connections between cell proliferation and cell death involves the group of compounds known as polyamines. Putrescine, spermidine and spermine are more usually associated with cell growth [6], but excessively elevated levels of polyamines may be the cause of cell death [7–9], a process which is, at least partly, independent of the oxidation of polyamines. More recently, it has been shown that the deregulated accumulation of polyamines is associated with the induction of apoptosis [10–13]. Polyamines, and spermine in particular, have also been shown to trigger caspase activation in cell-free models of apoptosis [14,15], suggesting a possible role for spermine in the transduction of a cell-death message. High cellular polyamine levels could therefore increase the sensitivity of tumour cells to therapeutic treatments inducing apoptosis. However, polyamines also have antioxidant capabilities [16], and are known to protect DNA against several kinds of stress [17,18]. Not surprisingly therefore, inhibition of polyamine synthesis can both protect [19–22] or sensitize cells exposed to death stimuli [23–25], depending on the cell type and the particular stimulus, suggesting a complex interaction between polyamines, cell growth and cell death.

Cell death is a complex event, and in a cell population exposed to toxic treatments, generally a mixture of types of cell death occurs. In the experiments described in the present paper we have focused our interest on the influence of polyamine levels on caspase activation in mouse fibroblasts. There is some evidence of caspase-independent apoptotic cell death [26], but the caspase

Abbreviations used: Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin; DFMO, D,L-α-difluoromethylornithine; MDL 72527, *N,N'*-bis-(buta-2,3-dienyl)butane-1,4-diamine.

[1] To whom correspondence should be addressed (e-mail cstefan@biocfarm.unibo.it).

cascade to date remains the best characterized event in apoptosis [1–4].

Mutant gyro (Gy) mice have disruption of the phosphate-regulating *Phex* gene, an X-chromosome-linked dominant trait [27]. The spermine synthase gene, located upstream from the *Phex* gene, is also extensively disrupted in Gy mice, this mutation being recessive, thus only males are affected [28]. Organs of Gy males have been shown to lack any significant spermine synthase activity as well as spermine content [28,29]. Fibroblasts derived from such Gy male mice also lack any significant spermine synthase activity and spermine content [29]. These cells are thus a valuable model to allow us to determine the requirement for spermine in the process of caspase activation.

EXPERIMENTAL

Materials

Polyamines, acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC), etoposide and all other biochemical reagents were Sigma products. Mouse monoclonal antibodies against caspase 3, caspase 9, Bcl-2, and p53 were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated anti-mouse IgG from Amersham was used as a secondary antibody.

Cells and treatments

Fibroblasts were derived from 14-day Gy male embryos and also from normal male littermate embryos, and immortalized, as reported previously [29]. Cells were grown in Dulbecco's modified Eagle's medium containing pyruvate and pyridoxine (GIBCO) supplemented with 2 mM glutamine, 10% (v/v) fetal-calf serum and 100 μg/ml each of penicillin and streptomycin, at 37 °C and 5% CO_2 in a humidified chamber. The fibroblasts were plated in 10-cm-diameter culture dishes with 7 ml of medium and, once confluence was reached, the cells were split again 1:12. Under our conditions, either cells derived from normal male littermates (N cells) or cells derived from Gy males (Gy cells) required about 5 days to reach confluence. In all the experiments, cells were grown for 2 days and then treated with inducers of apoptosis for the time indicated. The cells were subject to UV irradiation by leaving the dishes exposed to UV-C radiation (254 nm) for 10 min (80 J/m^2) at room temperature; control cells were similarly handled, but the lamp was turned off. In experiments involving the addition of polyamines, 1 mM aminoguanidine was added to the media in order to inhibit serum amine oxidases. Aminoguanidine did not elicit any effect on caspase activity (results not shown). Cell viability was evaluated by Trypan Blue exclusion and was quantified by counting 100 cells per dish, with the percentage cell death being defined as the percentage of cells that included the dye.

Caspase activity

The activity of caspase enzymes was measured by the cleavage of the fluorogenic peptide substrate Ac-DEVD-AMC, which represents a substrate for caspase 3 and other members of the caspase family [30]. At the end of the incubation with inducers of apoptosis, cells were washed in PBS, harvested in 0.4 ml of lysis buffer [20 mM Hepes (pH 7)/5 mM dithiothreitol/2 mM EDTA/0.1% CHAPS/0.1% Triton X-100/1 mM PMSF/aprotinin + pepstatin + leupeptin (1 μg/ml each)] and subjected to two cycles of freeze–thawing. The lysates were centrifuged for 10 min at 28000 *g* at 4 °C and the supernatant used to assay enzyme activity. A 10 μl portion of extract was incubated for 15 min at 37 °C in a final volume of 30 μl to determine caspase activity as previously described [31]. One unit is defined as the amount of enzyme activity cleaving 1.0 nmol of substrate/min under the standard conditions described. This activity will be referred to simply as 'caspase activity'.

Western blotting

The proteolytic processing of caspases 3 and 9 was detected in fibroblasts grown for 48 h and then exposed to etoposide for 24 h. The content of the Bcl-2 protein was assayed in cells grown for 48 h in the absence or presence of 5 mM DL-α-difluoromethylornithine (DFMO). The p53 protein was determined after 2, 6 and 16 h from etoposide addition or UV irradiation. At the end of these experiments, the cells were washed twice in PBS, scraped in lysis buffer [150 mM NaCl/0.25% sodium deoxycholate/1% Nonidet P40/1 mM NaF/1 mM EGTA/1 mM PMSF/aprotinin + leupeptin + pepstatin1 (μg/ml each)] and subjected to two cycles of freeze–thawing. After centrifugation at 28000 *g* for 30 min at 4 °C, the supernatants were assayed for protein concentration and then diluted in concentrated loading buffer [3% (w/v) SDS/10% (v/v) glycerol/4% (v/v) 2-mercaptoethanol/0.02% Bromophenol Blue/125 mM Tris/HCl (final concns.), pH 6.8] and boiled for 3 min. Aliquots corresponding to 50 μg of protein for each sample were analysed by SDS/12%-PAGE. Standard protein markers were used for molecular-mass calibration. After blotting, the nitrocellulose membrane was blocked for 1 h with 5% non-fat milk, washed with Tris-buffered saline, and probed for 1 h with a specific primary antibody. After washing, the membrane was then incubated for 30 min with the secondary antibody. Immunoreactive bands were revealed with an enhanced chemoluminescence kit (ECL®; Amersham).

Polyamine analysis

Cells were grown for 48 h, then were washed twice with PBS, harvested in 0.4 ml of chilled 0.3 M perchloric acid and subjected to two cycles of freeze–thawing. After centrifugation at 12000 *g* for 5 min at 4 °C, 0.3 ml of the clear supernatant was used for polyamine analysis, whereas the pellet was dissolved in 0.4 ml of 0.3 M NaOH for protein determination. Polyamines were separated and quantified by HPLC after derivatization with dansyl chloride [13]. When acetylpolyamines were also assayed, three plates per time point were pooled, and chromatographic analysis was performed as described in [32].

Data analysis

The results are expressed as means ± S.E.M. When applicable, statistical comparisons were made using Student's two-tailed paired *t* tests. Differences were considered significant at $P < 0.05$.

RESULTS

Fibroblasts derived from Gy males have previously been shown to lack significant spermine synthase activity and spermine content [29]. Gy fibroblasts and fibroblasts obtained from normal male littermates (N cells) grew at a similar rate. As expected, analysis of Gy cells used in the present study showed them to lack significant levels of spermine, the content being always lower than 0.5 nmol/mg of protein (Figure 1). Spermidine content, however, was significantly increased, probably to compensate for

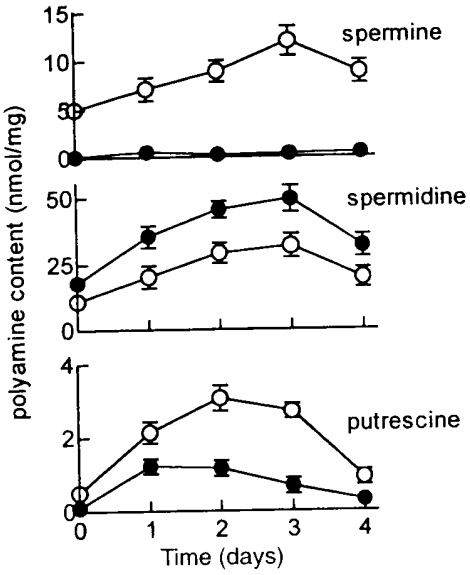

Figure 1 Polyamine content in transformed mouse fibroblasts obtained from a Gy male (●) or from a normal littermate (○)

Gy and normal (N) confluent cells were split in fresh medium to allow growth. At the indicated time, two plates for each cell type were independently analysed for polyamine content. The reported data are means ± S.E.M. of three determinations.

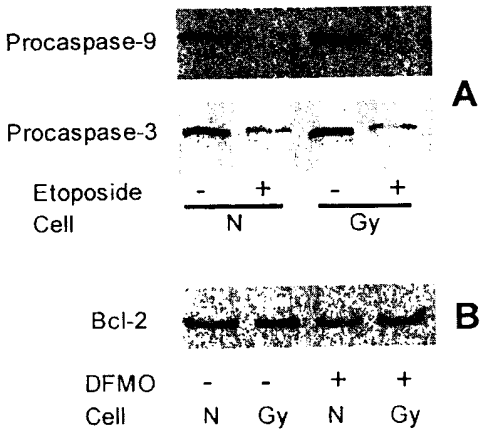

Figure 2 Caspase activation and Bcl-2 level in spermine-deficient Gy fibroblasts

(**A**) Normal (N) and Gy cells were grown for 48 h after plating, then treated with 20 μM etoposide or the vehicle alone (DMSO, 1 μl/ml). After additional 24 h of incubation, the cells were collected and the levels of pro-(caspase 9) and pro-(caspase 3) were determined. (**B**) The cells were grown for 48 h after plating in the absence or presence of 5 mM DFMO. Afterwards, the presence of the Bcl-2 protein in cell extract was assayed. Both experiments depicted in (**A**) and (**B**) were repeated twice with similar results. In all the experiments, aliquots of 50 μg of protein were analysed by Western blotting.

the lack of spermine. Putrescine was maintained at a low level, since it is immediately converted into spermidine. Thus, the total content of polyamines was not very different in the two cell lines.

Spermine is by far the most efficient polyamine in triggering caspase activation in whole cells [13] as well as in a subcellular model [14], suggesting a possible role of this tetra-amine in the transduction of a death message. Consequently, we initially tested the capability of Gy and N cells to undergo caspase activation. Gy and N fibroblasts were grown for 2 days after seeding, before being treated with etoposide, a topoisomerase II inhibitor, widely used as an antineoplastic drug and known to induce apoptosis in virtually all cell types [33]. After a further 24 h the cells were harvested and analysed for caspase activation by Western blotting. Figure 2(A) shows that etoposide triggered the processing of caspases 9 and 3, an initiator and an effector caspase respectively, evidenced by the reduction of the inactive proenzyme band, in both Gy and N cells.

Bcl-2 is one of the most powerful regulators of caspase activation [34], and we wanted to investigate whether the lack of spermine and, in general, the perturbation in polyamine distribution and content, would affect its expression (Figure 2B). The level of the Bcl-2 protein was similar in N and Gy cells, and only slightly and similarly decreased in both cell types following depletion of polyamines by the ornithine decarboxylase inhibitor DFMO (see below).

Even if the lack of spermine does not affect the ability of caspases to undergo activation, it could influence the degree of this activation, reflected by the enzyme activity within the cell. In order to study this point, cells were treated with two different inducers of apoptosis (etoposide and UV irradiation) and caspase activity assayed after 6, 15 and 24 h (Figure 3). During this time the activity of untreated control cells remained constant in both cell types (results not shown). In N and Gy fibroblasts, the time course of caspase activation was very similar following treatment with etoposide (Figure 3). In contrast, after UV irradiation, caspase activity was significantly higher in Gy cells, with Gy cells having twice the activity of N fibroblasts 24 h after UV irradiation (Figure 3). From these data, it appears that the lack of spermine selectively and strongly sensitizes the cells to DNA-damaging agents such as UV irradiation.

Changes in polyamine metabolism during apoptosis have been determined [35]. Polyamine levels had decreased in N and Gy fibroblasts incubated for 24 h after either treatment with

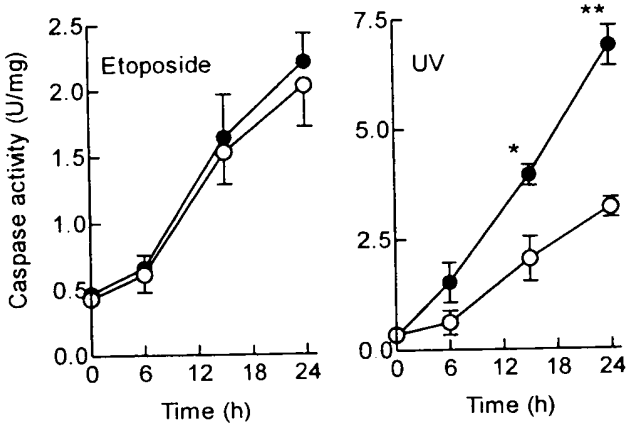

Figure 3 Caspase activity in normal (○) and Gy (●) fibroblasts undergoing apoptosis

Normal (N) and Gy cells were grown for 48 h after plating, then they were treated with 20 μM etoposide or exposed to UV irradiation (30 J/m^2) and again placed into the incubator. At the indicated times, the cells were collected and the caspase activity, cleaving the peptide sequence DEVD was assayed. During this time the activity in control cells did not show any significant change in either N or Gy cells. Note the different y-axis scales. Data are means ± S.E.M. for four estimations. Statistical analysis was used to compare Gy cells with N cells at the same time point (*$0.01 < P < 0.05$; **$P < 0.01$). Abbreviation: U, units.

Table 1 Effect of etoposide or UV irradiation on polyamine content

N and Gy fibroblasts were exposed to etoposide treatment (20 μM) or UV irradiation (80 J/m^2) after an initial growth period of 48 h. After further 24 of incubation from the treatment, the cells were harvested and analysed for polyamine content. Data are means ± S.E.M. of three determinations.

	Polyamine content (nmol/mg of protein)					
	N cells			Gy cells		
Treatment	Putrescine	Spermidine	Spermine	Putrescine	Spermidine	Spermine
None (control)	0.6 ± 0.3	29.2 ± 2.5	13.5 ± 1.7	0.9 ± 0.2	47.2 ± 3.2	< 0.4
Etoposide	1.1 ± 0.2	17.4 ± 2.3	8.1 ± 1.1	0.5 ± 0.2	19.8 ± 1.6	< 0.4
UV	2.3 ± 0.5	24.2 ± 3.1	10.7 ± 1.9	0.8 ± 0.1	36.7 ± 3.5	< 0.4

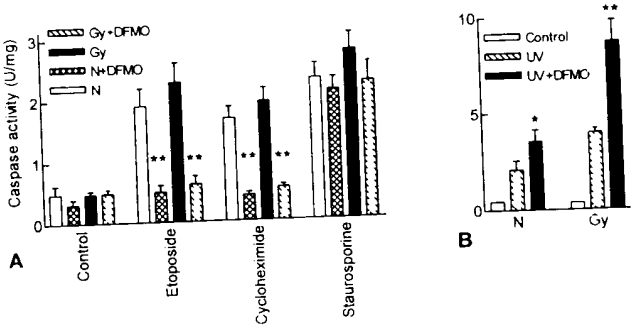

Figure 4 Effect of DFMO on caspase activity induced by different stimuli in normal (N) and Gy fibroblasts

The cells were grown for 48 h after plating in the absence or presence of 5 mM DFMO, then they were treated with: (**A**) nothing (control), 20 μM etoposide, 200 μM cycloheximide or 1 μM staurosporine. After 24 h, the cells were collected and caspase activity was assayed. In another experiment (**B**), N and Gy cells were exposed to UV irradiation (80 J/m^2), and caspase activity was assayed after 15 h incubation. For both (**A**) and (**B**), results are means ± S.E.M. obtained in four determinations and the *t* test was used to compare the caspase activity in presence of DFMO with the enzyme activity measured in absence of the drug ($^*0.01 < P < 0.05$; $^{**}P < 0.01$). Note the difference in the ordinate scales in (**A**) and (**B**). Abbreviation: U, units.

etoposide or exposure to UV. The decrease was especially pronounced in etoposide-treated cells (Table 1). In Gy cells, the spermine level was at or below the detection limit (0.4 nmol/mg of protein). No evidence of acetylpolyamine accumulation was observed within the 24 h incubation time of the experiment, in agreement with the finding that spermine/spermidine acetyltransferase activation is a late event in apoptosis [35].

Polyamine depletion by DFMO is reportedly able to inhibit or delay apoptosis induced by some stimuli [19–23], including etoposide [19]. DFMO does not decrease spermine content [6], and its effect in Gy cells should result in a more complete polyamine depletion compared with the N cells. Actually, following DFMO pretreatment (as described below in Figure 5) the spermine content of N cells was unchanged. To study the effect of polyamine depletion on caspase activation in N and Gy fibroblasts, the cells were pre-treated for 48 h with 5 mM DFMO and then treated with several inducers of apoptosis for a further 24 h. Figure 4(A) shows that caspase activity in cells treated with inducers of apoptosis, without DFMO pretreatment, was generally somewhat higher in Gy cells with respect to N fibroblasts when the inducers were etoposide, cycloheximide (an inhibitor of protein synthesis) or staurosporine (a protein kinase inhibitor); these differences, however, were not statistically significant. The effect of DFMO was similar in the two cell lines with no changes in the relative caspase activity between the Gy and N cells. Pretreatment with DFMO significantly inhibited the caspase activation that followed treatment with etoposide or cycloheximide ($P < 0.01$). On the other hand, DFMO did not affect the caspase activation that resulted from treatment with staurosporine.

A profound response to DFMO was seen in fibroblasts exposed to UV irradiation. In these experiments, caspase activity could not be accurately measured 24 h after the treatment, since pretreatment with DFMO strongly sensitized the cells to UV irradiation and caused massive cell death; the recovery of Gy cells pre-treated with DFMO was close to zero (see below). However, caspase activity could be measured 15 h following treatment (Figure 4B). DFMO pretreatment increased caspase activity in N cells by 1.7 times ($P < 0.05$), and even more so in Gy cells (2.2 times, $P < 0.01$). The combination of spermine deficiency and polyamine depletion in Gy cells caused a huge increase in caspase activation when triggered by UV irradiation (note the difference in the scales Figures 4A and 4B). As anticipated, this increased caspase activity was accompanied by increased cell death. DFMO pretreatment inhibited cell growth, as evidenced by the reduction in cell number with respect to control cells, without any significant effect on cell viability (Table 2). However, pretreatment with DFMO sensitized the cells to killing by UV irradiation. The lack of spermine *per se* was sufficient to sensitize the fibroblasts, since cell death was significantly higher in Gy cells compared with N cells ($P < 0.05$). The sensitivity to UV irradiation after DFMO pretreatment was particularly increased in Gy fibroblasts, since at 24 h following treatment, virtually all these cells were dead. On the other hand, when the trigger of cell death was etoposide, DFMO preserved the viability of both N and Gy cells, which is consistent with its inhibition of caspase activity shown in Figure 4(A).

In many cells, p53 is important in induction of apoptosis that follows DNA damage [36]. To determine if p53 was involved in the response of N and Gy fibroblasts, the cells were harvested at different times (2, 6 and 16 h) after etoposide or UV exposure, and p53 level was determined by Western blotting [31]. Neither etoposide or UV irradiation elicited any significant increase in p53 content at any time point (results not shown).

The abovementioned data show that polyamines, while protecting the cells against UV irradiation-induced apoptosis, are necessary for induction of apoptosis by etoposide. Actually, addition of 100 μM putrescine to DFMO-treated cells completely restored the polyamine content as well as the caspase activation that followed etoposide treatment (results not shown). Figure 5 shows that the preincubation of N fibroblasts with 5 mM DFMO for 48 h, the condition used in the previous experiments, caused

Table 2 Influence of DFMO on cell death induced by UV irradiation or etoposide

Normal (N) and Gy fibroblasts were grown for 48 h after plating in the absence or presence of 5 mM DFMO, then they were exposed to UV irradiation (80 J/m^2) or treated with 20 μM etoposide. After further 24 h, cell viability was assayed by Trypan Blue exclusion. Results are means ± S.E.M. of four determinations. The reported statistical analysis was used to compare the significance of DFMO pretreatment on cell death induced by each treatment (*$0.01 < P < 0.05$; **$P < 0.01$).

		N cells		Gy cells	
Treatment	DFMO	10^{-6} × Cell number	Cell death (%)	10^{-6} × Cell number	Cell death (%)
None (control)	–	3.96 ± 0.35	8.4 ± 1.4	3.48 ± 0.31	6.1 ± 1.7
	+	1.41 ± 0.22	9.3 ± 2.1	1.21 ± 0.13	10.6 ± 1.8
Etoposide	–	1.62 ± 0.21	64.3 ± 4.1	1.66 ± 0.24	71.0 ± 5.2
	+	1.38 ± 0.16	15.5 ± 3.1**	1.16 ± 0.17	24.4 ± 3.0**
UV	–	1.55 ± 0.27	36.9 ± 4.3	1.44 ± 0.09	57.5 ± 5.6
	+	1.22 ± 0.18	55.6 ± 5.9*	1.09 ± 0.16	98.3 ± 1.5**

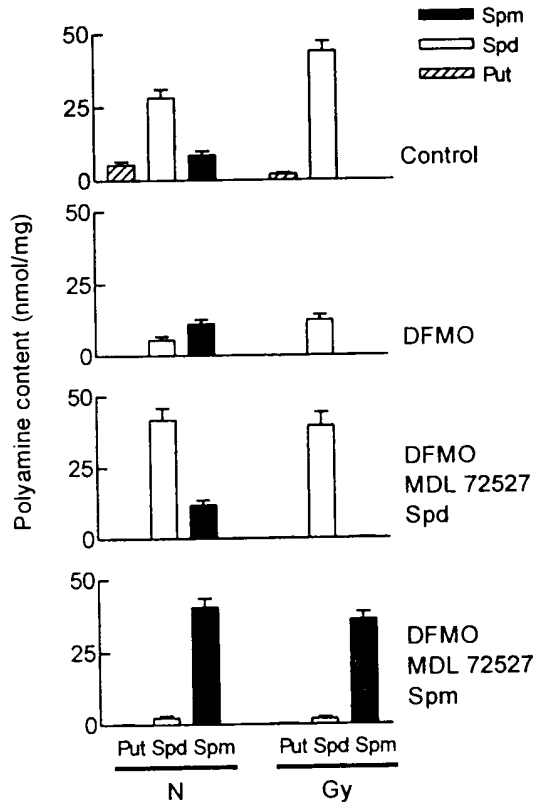

Figure 5 Effect of DFMO and polyamine addition on polyamine levels in normal (N) and Gy fibroblasts

N and Gy cells were grown for 48 h plus or minus 5 mM DFMO in the medium, then the cells were collected for polyamine analysis. Where indicated, DFMO-treated cells were supplemented for 15 h before their collection with 50 μM of a polyamine, spermidine or spermine, in the presence of 25 μM MDL 72527 to avoid interconversion and 1 mM aminoguanidine. Data are the mean ± S.E.M. of three determinations. Abbreviations: Spm, spermine; Spd, spermidine; Put, putrescine.

Table 3 Influence of polyamines on caspase activity triggered by etoposide

Normal (N) and Gy fibroblasts were grown for 48 h after plating in the absence or presence of 5 mM DFMO, then they were treated with 20 μM etoposide. Part of the DFMO-treated cultures was also supplemented for 15 h before etoposide with 50 μM of spermidine or spermine, in the presence of 25 μM MDL 72527, as indicated in Figure 5. Aminoguanidine (1 mM) was present in all samples. The cells were collected for caspase assay 24 h after etoposide administration. Data are means ± S.E.M. for results obtained in four determinations.

		Caspase activity (units/mg of protein)	
Treatment	DFMO	N cells	Gy cells
None (control)	–	0.41 ± 0.03	0.43 ± 0.04
	+	0.47 ± 0.05	0.36 ± 0.03
MDL 72527	+	0.51 ± 0.03	0.48 ± 0.06
+ Spermidine	+	0.52 ± 0.02	0.54 ± 0.06
+ Spermine	+	0.55 ± 0.06	0.57 ± 0.04
Etoposide	–	1.77 ± 0.13	2.16 ± 0.21
	+	0.64 ± 0.06	0.66 ± 0.07
+ MDL 72527	+	0.65 ± 0.06	0.70 ± 0.05
+ MDL 72527 + spermidine	+	1.83 ± 0.23	2.09 ± 0.19
+ MDL 72527 + spermine	+	1.58 ± 0.07	1.89 ± 0.12

the disappearance of putrescine and a large decrease in spermidine, but it did not significantly affect the content of spermine. In Gy cells, which have a higher basal spermidine content, the decrease in spermidine was less marked. As a consequence, the total level of polyamines (spermidine plus spermine) was not substantially different in N and Gy cells even after DFMO treatment. The observation that pretreatment with DFMO inhibits the effect of etoposide in N cells, that still contain spermine, as well as in Gy fibroblasts, lacking spermine, suggests that it is spermidine that is required for etoposide-induced caspase activation. However, it is not known if spermidine has an unique role or if it can be replaced with spermine. To answer this question we manipulated polyamine levels in N and Gy cells. DFMO-treated cultures were supplemented with 50 μM spermidine or spermine in the presence of *N*,*N*′-bis(buta-2,3-dienyl)butane-1,4-diamine (MDL) 72527, an inhibitor of the flavin-dependent polyamine oxidase, to avoid polyamine interconversion [37]. At this concentration, exogenous polyamines did not elicit significant cytotoxicity; however, 1 mM aminoguanidine was always present in the medium to inhibit serum amine oxidases [38]. In this way, a cell system containing high levels of a single polyamine with only traces of the others was developed. Gy cells pre-treated with DFMO and supplemented with MDL 72527 and spermidine contained virtually only spermidine (spermine was less than 0.4 nmol/mg of protein), whereas when either N or Gy cells were

replenished with spermine (plus MDL 72527), this polyamine was nearly the only cellular polyamine, because spermidine was reduced to less than 5% of its normal concentration. At present we do not know precisely the reason for this decrease in spermidine, but it is likely to be caused by dilution of existing pools by cell growth combined with spermine/spermidine acetyltransferase induction by the exogenous spermine that would cause spermidine acetylation [37] followed by excretion. Traces of acetylspermine were found in both N and Gy cells treated with spermine plus MDL 72527, but it was present at a barely detectable level and could not be measured accurately.

The DFMO-treated cells in which spermidine or spermine was replaced as described above were treated with etoposide, and caspase activity was assayed 24 h later. Table 3 shows that the replenishment of polyamine levels with either spermidine or spermine restored the ability of etoposide to trigger caspase activity in both cell types, indicating that, in this system, spermidine and spermine have interchangeable roles in the death pathway engaged by etoposide, and both are able to support the molecular event(s) leading to caspase activation.

DISCUSSION

Although polyamines have long been known to be essential for normal cell growth and differentiation [6], considerable evidence has emerged over the past few years that these cations also have a regulatory role in apoptosis. This conclusion is based upon two pieces of evidence. Firstly, inhibitors of polyamine synthesis can inhibit or delay apoptosis, suggesting an essential role for polyamines in this process [20–22,39,40]. However, caution is required here, since much in the literature has shown that polyamine depletion can both protect and increase the sensitivity of cells to death triggers, depending on the cell type and the stimulus [19,23–25,41–43]. Secondly, excessive polyamine levels are known to be associated with cell death. It has been known for many years that polyamines can be oxidized, generating toxic species [38,44], and also that this polyamine oxidation has been implicated in the mechanism of programmed cell death [39,45]. It is also evident that high polyamine levels are toxic *per se* [7–12], and that spermine can directly trigger caspase activation [13], suggesting a central role for this polyamine in apoptosis.

Transformed fibroblasts derived from Gy male mice represent a useful model where the influence of polyamines, and particularly of spermine, on cellular processes can be studied in detail. The chromosomal deletion in Gy males that results in the loss of spermine synthase activity also inactivates the *Phex* gene, but this gene, which encodes a membrane-bound endopeptidase involved in the regulation of phosphate metabolism, has a limited range of tissue expression [46,47]. Very recent work demonstrated that the *Phex* mRNA is absent in skin fibroblasts from both N and Gy males [48]. Such cells are devoid of spermine synthase activity and spermine content, but have an increased spermidine content, caused by the lack of its conversion into spermine and the up-regulation of *S*-adenosylmethionine decarboxylase [29]. Transformed fibroblasts obtained from Gy males are able to grow normally [29], indicating that spermine does not possess a unique and necessary role in cell proliferation and growth, but that spermidine can act as a substitute. We have studied the requirement for polyamines, in particular spermine, for caspase activation using these cells, and the data reported here define some points.

Firstly, spermine is not required for caspase activation, since caspase activity and processing can be triggered in the absence of spermine. Etoposide triggers the processing of the initiator caspase 9 and effector caspase 3, leading to the onset of caspase activity, in both N and Gy fibroblasts. We cannot completely exclude the possibility that the activation of some different caspase(s) is impaired in Gy cells, but this seems improbable, because, once activated, the caspase cascade leads to the direct activation of several members of the caspase family [4]. Also, the level of the Bcl-2 protein, a powerful regulator of caspase activation [34], is similar in N and Gy cells. Additionally, the lack of spermine actually increases the degree of caspase activation that follows exposure to UV irradiation, as discussed below. Evidently, even if spermine is a powerful trigger of caspase activation, at least in some cells [13], it is not necessary for the process in transformed fibroblasts.

Nevertheless polyamines are required for caspase activation triggered by some stimuli, including cycloheximide and etoposide. However, this is not a general characteristic of the fibroblasts, since staurosporine continues to activate caspases in polyamine-depleted cells. Therefore the requirement for polyamines in the process of caspase activation must be associated with the stimulus of apoptosis and the death pathway engaged. Some of the postulated mechanisms responsible for the anti-apoptotic effect of polyamine depletion by DFMO include inhibition of polyamine oxidation [39] and arrest of the cell cycle [49]. In the case of etoposide-induced caspase activation, the inhibiting effect of DFMO could also be correlated with the inactivation of topoisomerase II observed in polyamine-depleted cells [50], since it is known that topoisomerase II is required for the toxic effect of etoposide [51]. We have shown here that the polyamines have interchangeable role(s) in the pathway leading to caspase activation after etoposide treatment, since the replenishment of either spermidine or spermine levels alone is sufficient to allow the onset of caspase activity.

Another important observation is that, in UV-treated cells, the lack of spermine, and polyamine depletion in general, strongly increases the degree of caspase activation. In actual fact spermine deficiency alone is sufficient to render cells more susceptible to UV-induced damage, since caspase activity and cell death are considerably higher in Gy fibroblasts compared with normal cells. It thus appears that spermine has a protective function against the effect of UV irradiation. This function, differently from the role of spermine in caspase activation by etoposide and in cell growth, appears to be specific. The compensatory increase in spermidine content in Gy cells is not sufficient to keep the caspase activity triggered by UV irradiation at a level comparable with that in N cells. In contrast with other cell types [24,52], the depletion of putrescine and spermidine that follows DFMO treatment is sufficient to sensitize normal fibroblasts to UV irradiation, but it is the lack of spermine in the Gy fibroblasts that dramatically increases the sensitivity of the cells to UV irradiation. Since Gy cells also are much more sensitive to the cytotoxic effect of alkylating agents [29], it appears likely that spermine deficiency selectively impairs some process that follows DNA damage. While this paper was under revision, Nilsson et al. [48] reported that untransformed skin fibroblasts from male Gy mice exhibit decreased resistance to UV irradiation. Altogether, their and our data indicate that increased sensitivity to UV irradiation is a common feature of both transformed and untransformed cells lacking spermine. This increased sensitivity of Gy cells to UV-induced damage may be independent of the p53 status, since UV exposure, as well as etoposide, did not affect p53 level. However, at present we do not know if the lack of p53 response to DNA damaging agents is a characteristic of our embryo-derived fibroblasts or is brought about by their immortalization by a viral oncogene, which can lead to p53 inactivation [53].

In conclusion, we have shown here the differential effects of polyamine depletion on the process of caspase activation triggered by different death inducers. Contrasting data are reported in the literature about the effects of polyamine depletion on the cytotoxicities of anticancer treatments [19–24,41]. Our data suggest that the fate of a cell depleted of polyamines could be dependent on the specific effect that polyamines have on the pathway leading to caspase activation. Understanding of the functions of polyamines in the different death pathways resulting in caspase processing initiated by different death triggers could be of paramount importance to define the role(s) of polyamines in apoptosis. This knowledge should allow rationalization of the use of drugs depleting cellular polyamines in conjunction with cytotoxic treatments [54], in order to improve the results of cancer therapy.

This research was supported by funding from the Ministero dell'Università e della Ricerca Scientifica (MURST; fondi ex 40% and 60%) and Compagnia di San Paolo (Torino, Italy), and grants GM-26290 and CA-18138 from the NIGMS and the National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.

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Received 14 August 2000/19 December 2000; accepted 24 January 2001